Solution structure of the hypothetical protein Mth677 from *Methanobacterium thermoautotrophicum*: A novel α+β fold

FRANCISCO J. BLANCO,[1] ADELINDA YEE,[2] RAMÓN CAMPOS-OLIVAS,[1] ÁNGEL R. ORTIZ,[3] DEMIAN DEVOS,[4] ALFONSO VALENCIA,[4] CHERYL H. ARROWSMITH,[2] AND MANUEL RICO[5]

[1]NMR Group, Centro Nacional de Investigaciones Oncológicas, 28029 Madrid, Spain
[2]Division of Molecular and Structural Biology, Ontario Cancer Institute, and Department of Medical Physics, University of Toronto, Toronto, Ontario, Canada M5G 2M9
[3]Unidad de Bioinformática, Centro de Biología Molecular, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain
[4]Centro Nacional de Biotecnología, Consejo Superior Investigaciones Científicas (CSIC), Cantoblanco, 28049 Madrid, Spain
[5]Instituto de Química-Física "Rocasolano," Consejo Superior Investigaciones Científicas (CSIC), 28006 Madrid, Spain

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Abstract

The structure of Mth677, a hypothetical protein from *Methanobacterium thermoautotrophicum* (*Mth*), has been determined by using heteronuclear nuclear magnetic resonance (NMR) methods on a double-labeled ^{15}N-^{13}C sample. Mth677 adopts a novel α+β fold, consisting of two α-helices (one N terminal and one C terminal) packed on the same side of a central β-hairpin. This structure is likely shared by its three orthologs, detected in three other Archaebacteria. There are no clear features in the sequences of these proteins or in the genome organization of *Mth* to make a reliable functional assignment to this protein. However, the structural similarity to *Escherichia coli* MinE, the protein which controls that division occurs at the midcell site, lends support to the proposal that Mth677 might be, in *Mth*, the counterpart of the topological specificity domain of MinE in *E. coli*.

Keywords: *Methanobacterium thermoautotrophicum*; structural genomics; heteronuclear NMR; α+β new fold; MinE protein; cell division

Mth677 is a 91-residue hypothetical protein from the archaeon *Methanobacterium thermoautotrophicum*, whose proteome is the target of a pilot project to evaluate the feasibility of whole-genome-scale structural genomics (Christendat et al. 2000; Yee et al. 2002). Mth677 was initially selected in view of a well-dispersed ^{1}H-^{15}N HSQC nuclear magnetic resonance (NMR) spectrum and a consensus secondary structure prediction, indicating the presence of two helices and two β-strands, which could be arranged in a fold not previously described. Mth677 can not be related to any previously characterized protein, and its function is unknown. However, a BLAST search indicates that it is conserved, with high sequence homology to three hypothetical proteins present in the genomes of three other archaeal thermophiles (Fig. 1). These proteins are Ph0533 from *Pyrococcus horikossii*, Pab1365 from *Pyrococcus abyssi*, and Pf0383 from *Pyrococcus furiosus*. The sequences of the four proteins are about 25% identical and 50% similar, and the prediction of their secondary structure elements is essentially the same as that for Mth677. The four proteins have been classified as NOG06340, a nonsupervised orthologous group of proteins (von Mering et al. 2003). To

Reprint requests to: Manuel Rico, Instituto de Química-Física "Rocasolano," CSIC, Serrano 119, 28006 Madrid, Spain; e-mail: mrico@iqfr.csic.es; fax: 34-91-5642431.

Abbreviations: COG, cluster of ortholog groups; NOG, nonsupervised orthologous group; HSQC, heteronuclear single quantum correlation; HNHB, 3D NMR experiment directed to detect scalar cross-correlation between the amide and $H_{\beta\beta'}$ protons of a given residue.

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contribute to the effort in determining the structures of the non-membrane associated proteins of *Mth* and to look for clues that may unveil the functional role of this conserved protein from its structure, we have determined its three-dimensional structure in solution by NMR spectroscopy.

Results

Resonance assignment

Complete backbone (Fig. 2) and side chain assignments (^{1}H, ^{15}N, and ^{13}C) have been obtained for all Mth677 residues with the following exceptions: the nitrogen of Pro68, the ^{13}C carbonyl of the residue preceding Pro68, the $^{13}C_{\delta}$ and $^{13}C_{\zeta}$ of Phe89, the amine protons of lysines, and the protons of guanidinium groups of arginines. The assignment for the additional tag residues Gly-Ser-His is only partial. Nine pairs of methylene H_{β} resonances were stereospecifically assigned using HNHB and nuclear overhauser effect (NOE) information. A summary of the consensus chemical shift index, sequential and short-range NOEs, and amide proton exchange raw data is given in Figure 2. According to the whole set of NMR parameters, the elements of secondary structure are well delineated: two helices spanning residues 8–29 and 69–90 and two β-strands covering residues 36–46 and 49–60, in satisfactory agreement with the results of most predictive approaches. The assignments have been deposited in the BioMagResBank under accession number 5704.

Structure determination

Nine-hundred-sixty-six NOE-based distance restraints were obtained from the manual analysis of 2D NOESY, 3D ^{15}N NOESY-HSQC, and a 4D $^{13}C/^{13}C$-edited NOESY. Backbone dihedral angle restraints were obtained from the backbone and $^{13}C^{\beta}$ chemical shifts with the program TALOS (Cornilescu et al. 1999), in concert with $^{3}J_{HN\text{-}H\alpha}$ coupling constants. 16 χ_1 angle constraints were obtained for those residues with stereospecific methylene H_{β} assignment. Hydrogen exchange rates, together with preliminary structures, allowed the identification of 22 hydrogen bonds belonging to secondary structure elements. These bonds were included as 44 distance restraints (plus the corresponding 44 lower-bound distance restraints). These contraints were used to generate three-dimensional model structures of the protein, excluding the three tag residues at the N terminus. Preliminary structures were used for automatic assignment of 54 additional interresidue proton distance restraints with CANDID (Herrmann et al. 2002), which were included in the last round of structure calculation and refinement. The only peptide bond preceding a proline residue (Pro 69) was found to be in the *trans* conformation based on the chemical shifts of the proline carbons (Schubert et al. 2002) as well as on the sequential NOEs. Table 1 summarizes the structural

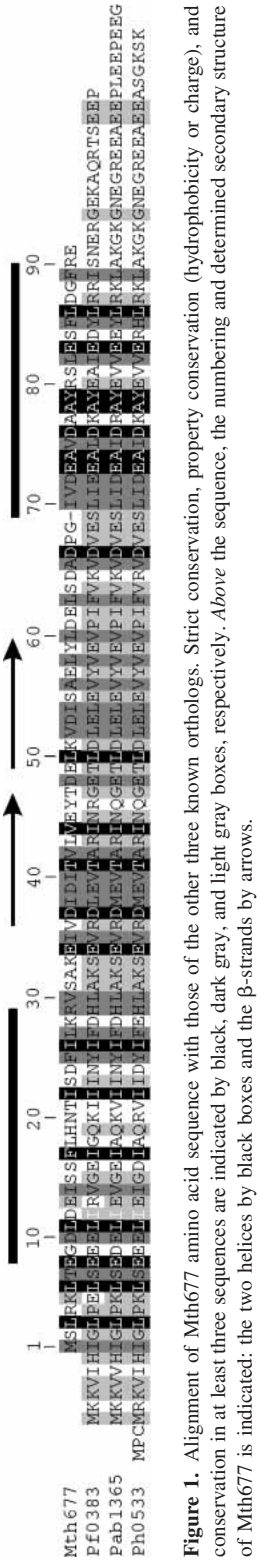

Figure 1. Alignment of Mth677 amino acid sequence with those of the other three known orthologs. Strict conservation, property conservation (hydrophobicity or charge), and conservation in at least three sequences are indicated by black, dark gray, and light gray boxes, respectively. *Above* the sequence, the numbering and determined secondary structure of Mth677 is indicated: the two helices by black boxes and the β-strands by arrows.

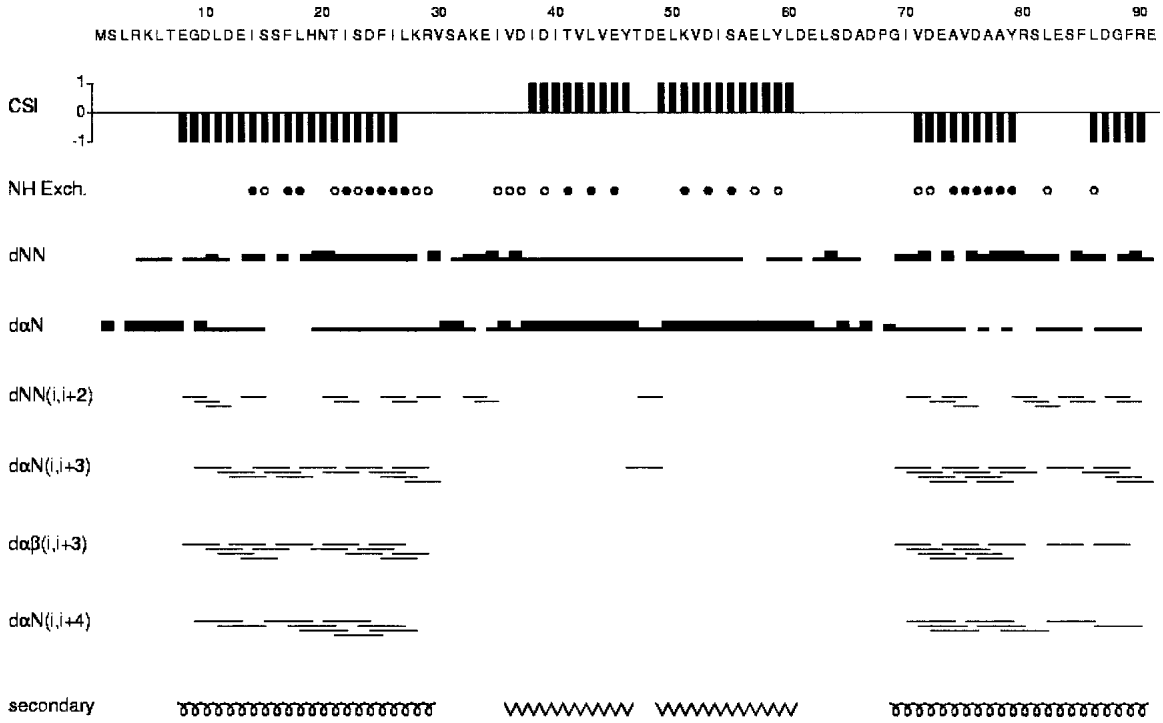

Figure 2. Summary of observed sequential and short-range NOEs, consensus chemical shift index (Wishart and Sykes 1994), and amide proton exchange data at 298 K and pH 7.0 vs. sequence number. The thickness of the sequential NOEs is a qualitative indication of their intensity. In the exchange plot, an open circle indicates that the amide proton of that residue remains in D_2O solution at pH 7 and T 298 K at least for 45 min, and a filled circle that the corresponding HN exchange took between 45 min and more than 15 h.

statistics of the final ensemble of 30 refined structures. The final ensemble of 30 refined structures has been deposited in the Protein Data Bank under accession code 1pu1.

The structure of Mth677

The overall topology of Mth677 is αββα (Figs. 2, 3). The two helices span residues 8–29 and 69–90 and are packed in an antiparallel way, making an angle of 140°. Two β-strands form a long hairpin from V36 to L60 that packs against the two helices. A single residue (D48), with average backbone dihedral angles typical of a γ-turn, and the only nonglycine residue with dihedral angle $\phi > 0$ in all the calculated conformers, connects the two antiparallel β-strands. This residue, together with the first two amino acids at the N terminus and the last two at the C terminus have heteronuclear ^{15}N $\{^1H\}$ NOEs smaller than 0.6 (data not shown), indicating high mobility at these sites. Other regions of the chain that connect the secondary structure elements adopt nonregular but well-defined structures, although with larger mobility than the secondary structure elements.

Table 1. *Structural statistics for the ensemble of 30 refined conformers of Mth677*

Restraints	
All	1273
Distance	
Intraresidue	213
Sequential ($\|i-j\| = 1$)	284
Medium range ($2 \leq \|i-j\| \leq 4$)	275
Long range ($\|i-j\| > 4$)	248
Hydrogen bonds	22 × 2
Dihedral angles	
ϕ	83
ψ	66
χ_1	16
Residual NOE violations per structure	
Number > 0.1 Å	0.5 ± 0.5
Maximum (Å)	0.27
Pairwise RMSD for residues 3–89 (Å)	
Backbone	0.75 ± 0.16
All heavy atoms	1.52 ± 0.14
Ramachandran map[a] for residues 3–89 (%)	
Residues in most favored regions	87.6
Residues in additionally allowed regions	11.0
Residues in generously allowed regions	0.3
Residues in disallowed regions	1.0

[a] Done with the program PROCHECK (Laskowski et al. 1996).

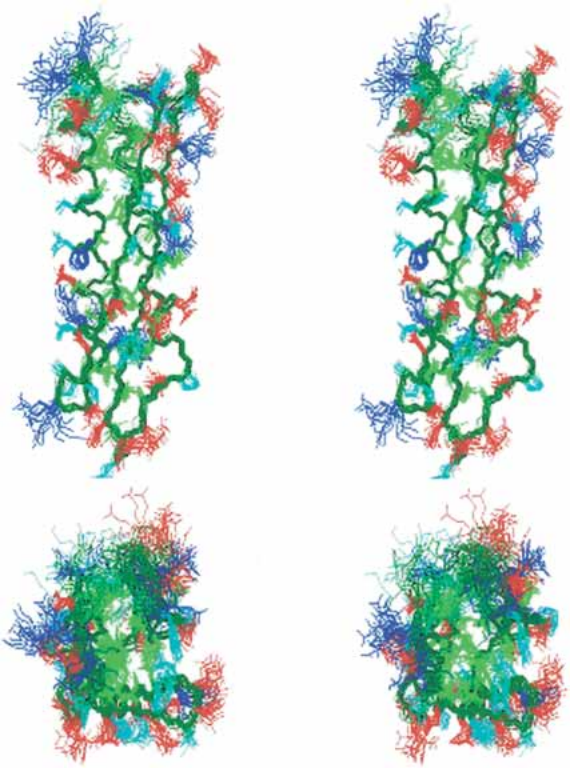

Figure 3. Stereo views of the solution structure of Mth677. (*Top*) Superposition (N, C^{α}, C′) of the 30 nuclear magnetic resonance (NMR) conformers showing the backbone in dark green, the hydrophobic core in pale green, the basic side chains in dark blue, the acidic ones in red, and the rest in cyan. (*Bottom*) View of Mth677 along its longitudinal axis where the predominantly acidic exterior of the protein can be seen. The figure was prepared with the program MolMol (Koradi et al. 1996).

The protein core is formed by 17 hydrophobic residues, all of which have more than 90% of their surface area buried (Fig. 3) and conserved in the four orthologs. The sequence of Mth677 has a large proportion of charged residues, most of them conserved, with their side chains located on the exterior of the protein surface or at the chain termini. These side chains are, in general, not well defined in the family of 30 NMR conformers, but inspection of the structure indicates that at least three ion pairs could be formed, one in each helix and the other one in the hairpin. Although the intrinsic thermal stability of a protein cannot be explained by a single factor, the tightly packed hydrophobic core and these ion pairs would certainly contribute to the thermal stability of Mth677. The melting of the structure monitored by circular dichroism at 222 nm is still not complete at 95°C (data not shown). From that curve, a melting temperature of about 80°C can be estimated.

The acidic amino acids (24% of the total) largely outnumber the basic ones (8%), yielding a theoretical pI of 4.0. The charge is asymmetrically distributed on the protein surface, with a greater density of negative charge on the exposed surface of the hairpin than on the helices' surface (Fig. 3 and Fig. 6, below). It is difficult to extract a conclusion from this finding, but it is tempting to think that it must be related to the protein function, although it is not obvious what the function can be for a protein with this charge distribution.

Search for structural homologs

A search for structural homologs of Mth677 was carried out using the structural alignment program MAMMOTH (Ortiz et al. 2002) on the ASTRAL domain database (Brenner et al. 2000; Chandonia et al. 2002), consisting of a set of 8000 protein domains derived from SCOP1.63 (Murzin et al. 1995; Lo Conte et al. 2002) and representing the known protein universe. Several statistically significant structural similarities were found, as defined by the MAMMOTH score, −lnP. For a given pair of structurally aligned proteins, this score quantifies the probability of finding an equal or larger number of structurally aligned residues only by chance. Scores > ~5 are indicative of a similarity at the fold level, while scores > ~7 clearly indicate that the two proteins share a common fold. The following domains were found above the cutoff of 7: d1bv1 (the birch pollen allergen, with a score of 9.66), d1gpma3 *Escherichia coli* (glutamine amidotransferase, 8.74), d1bcca2 (cytochrome bc1 complex from chicken, 8.01), d1e8ga1 (vanillyl-alcohol 2 oxidase, 8.01), d1ev0a (MinE topological specificity domain, 7.81), d1fa0a1 (yeast poliA polymerase, 7.27), and d1hr6a2 (alpha subunit of the yeast mitochondrial processing peptidase, 7.09). In most cases, Mth677 was matched to a supersecondary structure element forming part of a much larger domain, suggesting that the structural similarity observed in those cases was not reflecting an underlying functional similarity. On the other hand, $MinE^{TSP}$, the topological specificity domain, was an exception. $MinE^{TSP}$ is a small protein of only 58 residues functioning as a dimer (King et al. 2000). Both proteins share a very significant fraction of their structure. MAMMOTH finds that 51 out of the 58 residues of $MinE^{TSP}$ can be aligned to Mth677 with an RMSD of 3.87 Å (Cα). A similar search with DALI (Holm and Sander 1993; Dietmann et al. 2001) yielded five PDB structures with a *z* score >5. In all them, the fold of Mth677 was matched to a supersecondary structural element of a larger domain with more than two strands in the β-sheet. However, the structure of MinE protein is not selected by DALI as similar to Mth677, probably because of the different approaches used by both methods (contact map alignment in DALI and Cα trace superposition in MAMMOTH).

Discussion

Mth677 adopts a new fold

The arrangement of the secondary structure elements places Mth677 in the α and β proteins (α+β) class, defined by the

presence of mainly antiparallel β-sheets and segregated α and β regions in the Structural Classification of Proteins (Murzin et al. 1995). This class contains several folds and superfamilies, among them the fold and superfamily named head-to-tail joining protein W, represented by a single protein, the protein W of bacteriophage λ (Maxwell et al. 2002). This protein contains two antiparallel α-helices and a β-hairpin, like Mth677, but the two helices are packed against the opposite side of the hairpin (see Fig. 4). The two folds are different: They are mirror images of the arrangement of the elements of secondary structure (A. Murzin, pers. comm.). Although there is a significant structure similarity between MinE and Mth677, as evaluated by MAMMOTH, MinE is a homodimer whose global structure arises from the packing of secondary structure elements from each monomer (Fig. 5). The structure of Mth677 represents, then, a novel fold that is likely to be shared by its three identified orthologs.

Hints for Mth677 function from sequence similarity and genome organization in view of the structure

We can try to infer the function of the protein from its sequence alone, from the *Mth* genome organization and conservation among other genomes, and from structural details of the molecule.

Besides its three orthologs of unknown function, Mth677 is homologous to segments of proteins identified in the fruit fly, mouse, and human genomes, but with no functional annotation. The Web server STRING (von Mering et al. 2003), which uses conservation of neighborhood across genomes as the main criterion to predict functional associations, suggests as possible functions t-RNA binding and prefoldin or chaperonin cofactor. Considering the low pI of Mth677 and the distribution of charges on its surface, it is unlikely that this protein directly binds nucleic acids, although it could regulate the binding of other proteins. On the other hand, the archaeal prefoldin is a hetero hexameric protein that acts as a molecular chaperone stabilizing proteins and releasing them for subsequent chaperonin-assisted folding (Leroux et al. 1999), very different from the small monomeric Mth677. The three-dimensional structure of the protein allows us to look for local structural patterns associated with a particular function. The PINTS server (Stark and Russell 2003) was used to explore the presence of these patterns. Using different NMR models, some hits were found, but they were very diverse and had very high E values typical of negative matches, according to the authors.

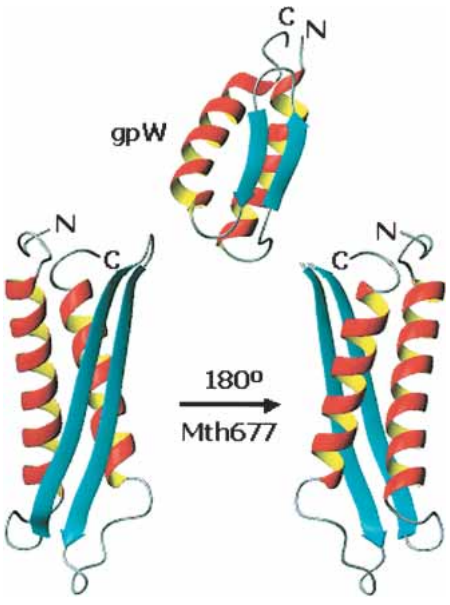

Figure 4. Comparison between the structures of bacteriophage λ protein W (*top*) and Mth677 (*bottom*). A 180° rotation along the longitudinal axis of the molecule is indicated to show that the arrangement of secondary structure elements in both proteins is different, and that they are mirror images. The figure was prepared with the program MolMol (Koradi et al. 1996).

Possible role of Mth677 in cell division

By applying the fold recognition program MAMMOTH (Ortiz et al. 2002), a match of part of the Mth677 structure (the N-terminal helix and the β-hairpin) with one subunit of the homodimer formed by the C-terminal domain of MinE was found. The MinE protein is involved in cell division and, more specifically, in securing that division takes place at the preferred midcell site. As a previous step to cell division, the tubulin-like protein FtsZ forms a circumferential ring (septum) at the division site. The complex formed by two other proteins, MinC and MinD, inhibits cell division by blocking the ring septum assembly. MinE forms an annular structure near the middle of the cell, suppressing the activity of the complex MinCD and allowing FtsZ ring formation at midcell (Margolin 2001). The 88-residue MinE protein of *E. coli* contains two separable functional domains. The 32-residue N-terminal counteracts MinCD activity, and the C-terminal domain ($MinE^{TSD}$) controls the topological specificity for midcell localization of MinE. $MinE^{TSD}$ forms in solution a homodimer, whose global structure consists of an antiparallel coiled coil, packed against one face of a four-stranded sheet (King et al. 2000).

As mentioned above, the N-terminal helix and the β-hairpin of Mth677 match very closely with one subunit of $MinE^{TSD}$, and even the C-terminal helix of Mth677 occupies the same position and orientation as the helix in the second subunit of the homodimer of $MinE^{TSD}$ (Fig. 5). Both proteins appear as more or less cylindrical when viewed from their longest axis (see Fig. 3, bottom, for Mth677; and Fig. 2 in King et al. 2000 for $MinE^{TSD}$). It is also worth noting the large negative electrostatic potential of both proteins and the similarity of its distribution, in particular the

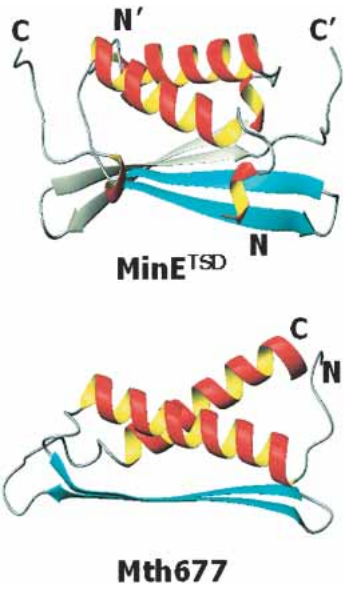

Figure 5. Comparison between the structures of *Escherichia coli* $MinE^{TSD}$ (*top*) and *Mth* Mth677 (*bottom*). $MinE^{TSD}$ is a noncovalent dimeric protein, in which the chain termini of each monomer have been labeled with primed or unprimed letters. The secondary structure elements that match the ones in Mth677 (one hairpin of one monomer and the helix of each of the two monomers) are colored as in Mth677, and the rest of the chains are gray.

large negative potential on the solvent-exposed surface of the β-sheet (see Fig. 6). It is reasonable to speculate that both shape and charge might be required to form the annular structure by side-by-side juxtaposition of a number of these modular proteins and/or by interaction with the internal side of the membrane. In that case, the annular structure formed by Mth677 and the homodimeric $MinE^{TSD}$ would be very similar, and then Mth677 would be the equivalent of $MinE^{TSD}$ in *Mth*. A fact in favor of that proposal is that there is no homolog of $MinE^{TSD}$ in the genome of *Mth*, and that homologs of MinD and FtsZ are, however, found. On the other hand, no clear homologs of MinC and of the N-terminal domain of MinE were found. The proposal that Mth677 might be, in *Mth*, the counterpart of $MinE^{TSP}$ in *E. coli* needs to be further confirmed. A right step in that direction would be to observe the consequences of deleting the implied gene on cell division.

Materials and methods

Sample preparation and purification

Mth677 was cloned in plasmid pET15b with a $(His)_6$ tag and a thrombin cleavage site at its N terminus. The plasmid was transformed into *E. coli* BL21–(Gold λDE3) cells cotransformed with a plasmid encoding three transfer RNAs for rare *E. coli* codons and grown at 37°C until the $OD_{600\ nm}$ reached 0.6. After protein expression induction with 1 mM isopropyl β-D-thiogalactoside, the temperature was reduced to 15°C and the cells were allowed to grow overnight before harvesting. Frozen cell pellets were thawed in 500 mM NaCl/20 mM Tris/5 mM imidazole (pH 8) and lysed by sonication. The protein was extracted by batch Ni^{2+} affinity chromatography (Qiagen), and the affinity beads were washed three times with five column volumes of 500 mM NaCl/20 mM Tris/30 mM imidazole (pH 8); the protein was eluted with five column volumes of the same buffer plus 500 mM imidazole. The His tag was removed by cleavage with thrombin and the protein purified by anion exchange chromatography using DEAE Sepharose. The purified protein was concentrated, and the buffer was exchanged by ultrafiltration and dilution/reconcentration. The final 94 residue long polypeptide analyzed here contains the sequence of Mth677 protein plus three extraneous residues (GSH) at its N-terminus, coming from the thrombin cleavage site. Equilibrium sedimentation analysis indicates that Mth677 is a monomer under the conditions used for the acquisition of the NMR spectra (data not shown). Its circular dichroism spectrum is typical of a protein with mixed α and β secondary structure. Three labeled samples were prepared growing bacteria in M9 minimal medium supplemented with $^{15}NH_4Cl$ or with $^{15}NH_4Cl$ and $[^{13}C_6]$ glucose U-^{15}N, U-^{13}C,^{15}N, and U-^{13}C,^{15}N fully exchanged in ^2H_2O.

NMR spectra

NMR experiments were recorded on a Bruker AVANCE 600 spectrometer with a triple resonance (^1H, ^{15}N, and ^{13}C) z-gradient probe at 298 K. NMR samples were prepared with a protein concentration of approximately 1.2 mM in 25 mM sodium phosphate buffer (pH 7.0), 0.45 M NaCl, 10 mM DTT, 20 μM $ZnCl_2$, 1 mM benzamidine, and a protease inhibitor mixture (Roche Molecular Biochemicals). Some spectra were also recorded at 278 K to assign overlapping resonances.

For the sequential assignment of backbone resonances HNCACB, CBCA(CO)NH, HNHA, and HNCO experiments (Bax and Grzesiek 1993) were acquired and analyzed with reference to the ^1H-^{15}N HSQC spectrum. Figure 7 shows the ^1H-^{15}N HSQC spectrum as a sample of the spectral quality. The following experiments were performed for the assignment of nuclear reso-

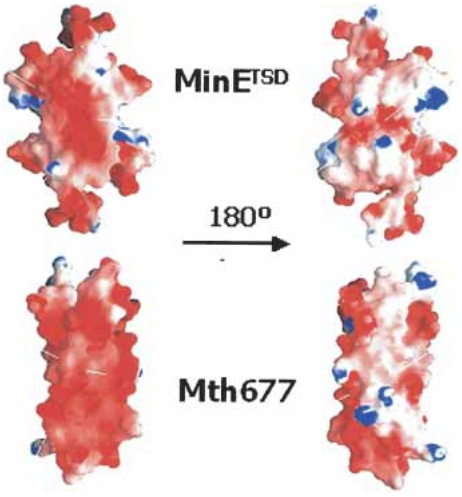

Figure 6. Comparison between the electrostatic potential surfaces of $MinE^{TSD}$ and Mth677. A highly negative electrostatic potential is common to both molecules on the solvent-exposed surface of the hairpin(s), shown at the *left*. This orientation is the same as in Figure 3.

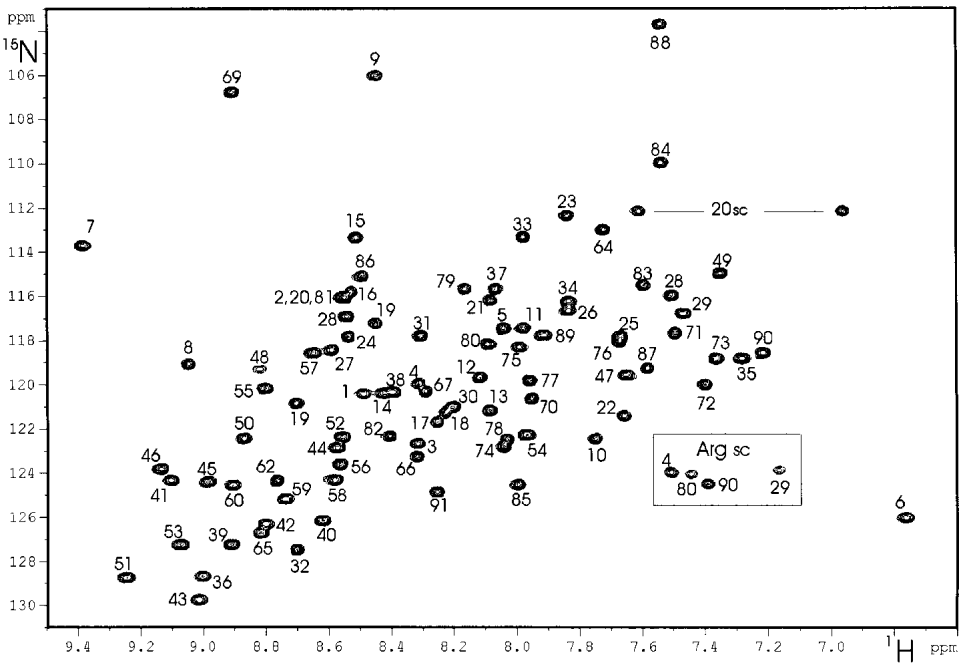

Figure 7. One bond $^{1}H–^{15}N$ single quantum correlation of nuclei ^{1}H and ^{15}N in Mt677 with assignments.

nances in side chains: HNHB (Bax et al. 1994); 2D ^{1}H-^{1}H NOESY and TOCSY with mixing times of 120 and 80 msec, respectively; 3D ^{15}N edited NOESY and TOCSY (same mixing times as the 2D) (Bax et al. 1990; Grzesiek et al. 1993); two different CT-^{1}H-^{13}C HSQC, optimized for correlations involving either aliphatic or aromatic protons; 3D (H)C(CCO)NH (15.2 msec carbon mixing time using the DIPSI sequence); and HCCH-TOCSY (Bax et al. 1994; 18 msec mixing time). The aromatic side chains were assigned to specific residues from their NOEs with the βCH_2 groups and the backbone amide protons.

Spectra were processed with the program NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson and Blevins 1994). Chemical shifts were measured relative to internal TSP for ^{1}H and calculated for ^{15}N and ^{13}C using the giromagnetic constant ratios (Wishart et al. 1995).

Structure determination

The NMR constraints were used as input for the torsion angle dynamics program CYANA (Güntert et al. 1997) to generate three-dimensional model structures of the protein, excluding the three tag residues at the N terminus. The structures were refined by energy minimization with the AMBER 7.0 package (Case et al. 2002). In this step, all distance restraints involving methyl and nonstereospecifically assigned protons were used with an average of r^{-6}, and additional constraints to keep the planarity of the backbone peptide bonds and the chirality of the amino acids were included.

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